Inhibitory Effect of Taraxci Herba Methanol Extract on Pro-inflammatory Mediator in Lipopolysaccharide - Activated Raw 264.7 cells

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Taraxci Herba (TH; Pogongyoung in Korean) has been used in traditional oriental medicine for the treatment of various ailments. The biological activity of this plant is not yet evaluated systematically. This study was conducted to evaluate the inhibitory effects of TH on the production of nitric oxide (NO) and the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated Raw264.7 cells. The aim of the present work is to investigate a potential anti-inflammatory activity of TH. The Raw264.7 cells were cultured in DMEM medium for 24 h. After serum starvation for 12 h, the cells were treated with TH for 1 h, followed by stimulating NO production with LPS (2 μg/ml). As a result of this study, TH inhibited the levels of NO, PGE2, TNF-α, IL-6 and IL-1β, and the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) activated by LPS. These inhibitory effects were mediated through the inhibition of phosphorylation of inhibitory kappa B (IκB). These findings showed that TH could have some anti-inflammatory effects.

Key words : Taraxci Herba, iNOS, COX-2

Introduction

Taraxci Herba (TH; Pogongyoung in Korean) has been used in traditional oriental medicine for the treatment of various ailments. It is used internally for treatment of disturbances in bile flow, inflammatory conditions of the efferent urinary tract, and dyspepsia. It is also used for liver and gallbladder disorders, hemorrhoids, congestion in the portal system, gout, rheumatic disorders, eczema, and other skin disorders\textsuperscript{1}. Its therapeutic value has been tested in various systems, but the biologically active agent has not been identified\textsuperscript{2}.

LPS-activated macrophages have usually been used for evaluating the anti-inflammatory effects about various materials\textsuperscript{3}. Lipopolysaccharide (LPS) is a prototypical endotoxin derived from Gram-negative bacterial membrane and is the initial stimulus leading to induce septic shock syndrome\textsuperscript{4}. LPS can directly activate macrophages, endothelial\textsuperscript{5}.

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cells and the complement-triggering production of inflammatory mediators, such as nitric oxide (NO), tumour necrosis factor-α (TNF-α), interleukins (ILs) and leukotrienes\textsuperscript{5}.

Nitric oxide (NO) is a gaseous molecule synthesized from L-arginine in the presence of nitric oxide synthase (NOS) enzyme and is involved in inflammation, immune function, bone metabolism, and apoptosis\textsuperscript{5}. Three NOS isoforms, neural NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) are known\textsuperscript{5}. Inducible nitric oxide synthase (iNOS) is associated with inflammation, and its reaction product NO is involved in various diseases, such as psoriasis and atopic inflammation\textsuperscript{5}. TNF-α is another major mediator in inflammatory responses, inducing innate immune responses by activating T cells and macrophages, and stimulating secretion of other inflammatory cytokines\textsuperscript{5}. In LPS-inducible tissue injury and shock, TNF-α is therefore thought to be a principal mediator\textsuperscript{5}. IL-1β is another inflammatory cytokine, which is found in the circulation following Gram-negative sepsis, and a mediator of the host inflammatory response in innate immunity\textsuperscript{5}. IL-6 is also an inflammatory cytokine mainly synthesized by macrophages, and plays a role in the acute phase response\textsuperscript{5}. And cyclooxygenase-2 (COX-2) is also involved in pathological mechanism of chronic inflammation.
COX-2, an inducible isoform of cyclooxygenase, is a key enzyme that synthesizes PGE2 in response to inflammatory stimuli such as LPS\textsuperscript{18}. Thus, pharmacological reduction of LPS-inducible inflammatory mediators (for example NO, TNF-α and ILs) is regarded as one of the essential conditions to alleviate a variety of disorders caused by activation of macrophages\textsuperscript{16}. This study evaluated the effect of methanol extract of TH on the regulatory mechanism of NO, cytokines and prostaglandin E2 (PGE2) in the LPS-activated Raw264.7 cells.

Materials and Methods

1. Reagents

LPS (Escherichia coli 026:B6) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium were obtained from Sigma (St. Louis, MO, USA). The fetal bovine serum (FBS) and antibodies were purchased from Gibco/BRL (Eggenstein, Germany). The antibodies were obtained from BD Bioscience (San Jose, CA, USA), Cayman (Ann Arbor, MI, USA) and Zymed (San Francisco, CA, USA), and the NC paper used was Schleicher & Schuell (USA). The TNF-α, IL-6 and IL-1β ELISA Kits were purchased from Pierce endogen (Rockford, IL, USA).

2. Preparation of Methanol Extract of Taraxi Herba

TH (300 mg) was prepared by extracting with 1000 ml of methanol (MeOH) at room temperature for 24 h. The extract was filtered through a 0.2 μm filter (Nalgene, New York, NY, USA). The amount of TH MeOH extract was estimated by the dried weight of lyophilized MeOH extract of TH. The yield of lyophilized TH MeOH extract was 9.17%. The lyophilized extract was stored at -20°C until needed.

3. Cell Culture

Raw264.7 cell, which is a murine macrophage cell line (KCLRF, Korean Cell Line Research Foundation, Seoul, Korea), was cultured in Dulbeco’s modified Eagle’s medium (DMEM, Gibco, Germany) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. For all experiments, the cells were grown to 80-90% confluence, and were subjected to no more than 20 cell passages. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The Raw264.7 cells were plated at a density of 2-3×10⁴/ml and pre-incubated at 37°C for 24 h. After serum starvation for 24 h, the cells were exposed to either LPS (2 μg/ml) or LPS+TH for 24 h. TH was dissolved in DMSO + medium (EMEM, Cambrex Bio Science, MD, USA) and added to the incubation medium 1 h prior to adding the LPS.

4. Assay of NO Production

The level of NO production was monitored by measuring the nitrite concentration in the cultured medium. Briefly, the samples were mixed with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthyl-ethylendiamine dihydrochloride and 2.5% phosphoric acid) and incubated for 10 min at room temperature in dark. The absorbance was measured at 540 nm using a Titerdek MultiTask automatic ELISA microplate reader (Model MCC/340, Huntsville, AL, USA).

5. Cell Viability

The Raw264.7 cells were plated at a density of 1×10⁴ cells/well in a 24 well plate to determine the cytotoxic concentrations of TH. Cells were serum-starved for 24 h, and then treated with LPS (2 μg/ml) or LPS+TH for the next 24 h. After incubation of the cells, viable cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml, 4 h). The media were then removed, and produced formazan crystals in the wells were dissolved by addition of 200 μl dimethylsulphoxide. Absorbance was measured at 540 nm using a Titerdek MultiTask automatic ELISA microplate reader (Model MCC/340, Huntsville, AL, USA). Cell viability was defined relative to untreated control cells (that is viability (% control):100(absorbance of treated sample)/(absorbance of control)).

6. Immunoblot Analysis

The cells were lysed in the buffer containing 20 mM Tris (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 mg/ml leupeptin. The total cell lysate was prepared by centrifuging the cells at 10,000-g for 10 min and collecting the supernatant. The expression of iNOS, COX-2 and p-IκB was immunologically monitored with the total lysate fraction using anti-rabbit iNOS, COX-2 and p-IκB antibodies, respectively. The bands for the iNOS, COX-2 and p-IκB proteins were visualized using ECL western blotting detection reagents (Amersham Biosciences, New Jersey, USA) according to the manufacturer’s instructions.

7. Measurement of Cytokines Production

For the cytokine immunoassays, the cells (1×10³/ml) were pre-incubated with TH for 1 h and further cultured for 24 h with 2 μg/ml of LPS in 24-well plates. The supernatants were removed at the allotted times and the level of TNF-α, IL-6 and IL-1β production was measured using an ELISA Kit (Pierce endogen, Rockford, IL, USA) according to the manufacturer’s
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briefly, 50 μl of biotinylated antibody reagent and the samples were added to the anti-mouse TNF-α, IL-6 and IL-1β pre-coated 96-well strip plates. The plates were covered and kept at room temperature for 2 h and washed three times in a prepared washing buffer. This was followed by the addition of 100 μl of Streptavidin-HRP Concentrate. After 30 min incubation at room temperature, the wells were washed three times, and 100 μl of TMB Substrate Solution was then added and developed in the dark at room temperature for 30 min. The reaction was quenched by adding 100 μl of TMB Stop Solution, and the absorbance of the plates was measured at 450 nm to 550 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-6 and IL-1β in serial dilutions. The level of TNF-α, IL-6 and IL-1β was quantified from standard curve.

8. Measurement of PGE2 Production

TH was treated into culture medium 1 h before the addition of 2 μg/ml LPS. LPS-treated cells were further cultured with vehicle or TH for 24 h. The cultured medium was collected and assayed with ELISA kit (R&D Systems, Minneapolis, MN, USA). Cultured medium was incubated in goat anti-mouse IgG coated plate with acetylcholinesterase linked to PGE2 and PGE2 monoclonal antibody for 24 h at 4°C. The plate was emptied and rinsed five times with wash buffer contained in the kit. And then, 200 μl of substrate reagent was added to each well and incubated for 1 h at 37°C. The developed plate was read at 405 nm and the PGE2 concentration of each sample was determined according to the standard curve.

9. Scanning Densitometry

Scanning Densitometry of the immunobots was performed with a UVP Epi Chemi Bioimaging System (UVP, Inc., Upland, CA).

10. Statistical Analysis

The data were expressed as a mean ± S.D. of the results obtained from a number of experiments. One-way analysis of variance (ANOVA) was used to assess the significant differences between the treatment groups. For each significant effect of treatment, the Tukey test was used to compare the multiple group means. A P value < 0.05 was considered significant.

Results

1. Taraxi Herba Inhibits LPS-Activated Nitric Oxide

The inhibition of NO production by TH was investigated by measuring the level of NO production in Raw264.7 cells treated with 0.01, 0.03, 0.1 and 0.3 mg/ml of TH. As shown in Fig. 1A, the level of NO production decreased TH treatment in a concentration-dependent manner compared with the LPS group. In the 0.01, 0.03, 0.1 and 0.3 mg/ml of TH group, the level of NO was significantly inhibited at 24 h (Fig. 1A). Cell viability was measured at 0.01 ~ 0.3 mg/ml of TH for 24 h using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in order to determine if the concentrations of TH used in this study induce cytotoxicity. The results showed that TH concentration did not have any toxicity after 24 h incubation (Fig. 1B).

![Graph A] (Nitric Oxide (μM))

![Graph B] (Cell Viability (% of Control))

Fig. 1. NO release and the cytotoxicity by Taraxi Herba in LPS-activated Raw264.7 cells. The NO concentration (A) in the culture medium and cytotoxicity (B) were measured for 24 h. The data represent the mean ± SD of three separate experiments. One-way ANOVA was used to compare the multiple group means and followed by Tukey test (** significant compared with the control, #P<0.01, ## significant compared with the LPS alone, ###P<0.001). TH, Taraxi Herba LPS, lipopolysaccharide.

2. Inhibitory Activities of Taraxi Herba on LPS-Activated iNOS and COX-2 Expression

The expression level of iNOS protein in the cytosol fraction was examined using immunoblotting analysis. The iNOS protein expression was reduced by TH. As shown in Fig. 2, 0.1 mg/ml of TH strongly suppressed the induction of iNOS by LPS and 0.3 mg/ml TH markedly reduced the protein levels of iNOS expression. The iNOS plays a key role in the development of inflammation against infectious agents by producing an excess amount of NO.
We next investigated whether TH might affect the levels of COX-2 expression. As shown in this experiment, the COX-2 protein was strongly induced by LPS. 0.3 mg/ml of TH slightly suppressed the induction of COX-2 (Fig. 2).

Fig. 2: The inhibitory effects of Taraxci Herba on iNOS and COX-2 induction by LPS. The protein levels of iNOS and COX-2 were monitored 16 h after treatment with LPS alone or in combination with TH (0.03, 0.1 and 0.3 mg/ml). The relative levels of iNOS and COX-2 were measured by scanning densitometry (B, C). Actin is used as a loading control. TH, Taraxci Herba; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

3. Taraxci Herba Inhibits LPS-Activated pro-inflammatory Cytokines

We analysed the inhibitory effects of TH on pro-inflammatory cytokines, including TNF-α (Fig. 3A), IL-1β (Fig. 3B) and IL-6 (Fig. 3C). These cytokines are involved in a variety of immunological functions as well as interaction with a variety of target cells. Production of the cytokines was measured by using ELISA Kit in the media of Raw264.7 cells treated with LPS (2 ug/ml) alone or in combination with TH. As show in Fig. 3, TH treatment concentration-dependently inhibited production of the cytokines at 24 h incubation.

Fig. 3: Inhibition of Taraxci Herba on LPS-activated cytokines production. Raw264.7 cells were cultured with LPS (2 ug/ml) in the presence or absence of TH for 24 h to determine the levels of TNF-α (A), IL-1β (B) and IL-6 (C). The cultured media were collected and directly assessed for cytokines. The data represent the mean ± SD of three separate experiments. One-way ANOVA was used to compare the multiple group means followed by Tukey test (*p < 0.05, **p < 0.01, ***p < 0.001). LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

4. Taraxci Herba Inhibits LPS-Activated PGE2 Release

To assess whether TH could inhibit LPS-induced PGE2 production in the Raw 264.7 cells, we assayed PGE2 concentrations in the culture media of cells pretreated with TH and subsequently treated with LPS for 24 h. The PGE2 production induced by 2 ug/ml LPS was significantly inhibited by pretreatment with TH in concentration-dependent manner (Fig. 4).

5. Inhibitory Activities of Taraxci Herba on IκB Phosphorylation

In order to determine if TH can directly affect phosphorylation of IκB in macrophage cells, the level of p-IκB
protein expression was assessed immunochemically in RAW264.7 cells incubated with or without TH. LPS alone increased the phosphorylation of IkB level. However pretreatment of 0.1 and 0.3 mg/ml of TH reduced the LPS-inducible p-IkB expression level (Fig. 5). These findings suggest that TH inhibited LPS-inducible IkB phosphorylation. Thus TH can prevent NF-κB translocation from cytosol to nuclear by inhibiting IkB phosphorylation.

![Graph](image1)

Fig. 4. Inhibition of LPS-activated PGE2 production by Taraxic Herba. RAW264.7 cells were cultured with LPS (2 μg/ml) in the presence or absence of TH for 24 h to determine the level of PGE2. The cultured medium was collected and directly assayed for PGE2. The data represent the mean ± SD of three separate experiments. One-way ANOVA was used to compare the multiple group means followed by Tukey test (**p < 0.01, *p < 0.05, **p < 0.01). PGE2, prostaglandin E2.

![Graph](image2)

Fig. 5. Inhibition of LPS-activated p-IkB protein expression by Taraxic Herba. The protein levels of p-IkB were monitored 15 min after treatment with LPS alone or in combination with TH (0.03, 0.1 and 0.3 mg/ml). The relative levels of p-IkB were measured by scanning densitometry (A). Actin is used as a loading control p-IkB, phospho-inflammatory kaza B.

**Discussion**

Inflammation is a host response to harmful stimuli. The pathology of inflammation is initiated by multiple processes triggered by microbial pathogens such as LPS, which is a prototypical endotoxin. LPS can directly activate macrophages, which trigger the production of pro-inflammatory mediators, such as NO, TNF-α, ILs and leukotrienes. Thus, pharmacological reduction of LPS-inducible pro-inflammatory mediators is regarded as one of the essential conditions to reduce a variety of disorders caused by activation of macrophages.

In this study, we showed that Taraxic Herba (TH) could modulate the regulatory mechanism of nitric oxide (NO), cytokines and PGE2 in the lipopolysaccharide (LPS)-activated Raw264.7 cells. TH inhibited the levels of NO, PGE2, TNF-α, IL-6 and IL-1β, and the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) activated by LPS. These inhibitory effects were mediated though the inhibition of inhibitory kappa B (IκB) phosphorylation.

NO is a radical produced from L-arginine by nitric oxide synthases (NOSs), and an important cellular second messenger. The modulation of iNOS-mediated NO release is one of the major contributing factors during the inflammatory process. Moreover, NO derived from macrophage is an important host defense and microbial and tumor cell killing agent, as well as a regulator of pro-inflammatory genes in vivo. At adequate concentrations of NO, it can generate and modify intracellular signals, thereby affecting the function of immune cells, as well as tumor cells and resident cells of different tissues and organs. Macrophages constitute one of the first lines of host defense against microbial infections based on their abilities to produce NO and reactive oxygen species. Although iNOS-derived NO primarily acts as an antimicrobial and tumoricidal agent, uncontrolled release of NO has also been implicated in inflammatory destruction of the target tissue in infection. Therefore, for above reasons, the modulation of iNOS-derived NO release appears as plausible candidate for therapeutic optimization of protective immunity and prevention of detrimental effects of inflammation. Therefore, we investigated in this study whether TH decreased the level of LPS-induced NO production and iNOS expression. In our study, it showed that TH effectively reduced the NO concentration and blocked the induction iNOS protein.

Next, we investigated whether TH might affect the levels of COX-2 expression and PGE2 content. Cyclooxygenase (COX) is the key enzyme in the synthesis of prostaglandins from arachidonic acid. Two isofoms of COX are known: cyclooxygenase-1 (COX-1), a constitutively expressed enzyme responsible for the production of prostaglandins with general housekeeping functions and COX-2, an inducible isofom of
COX-2 is a key enzyme of catalyzing the production of prostaglandin in response to pro-oxidant and pro-inflammatory stimuli. COX-2 plays a major role in the development of inflammation by NF-kB activation. Many of the side effects of NSAIDs have been ascribed to the suppression of COX-1-derived prostanoid production. By contrast, the specific inhibitor of COX-2 provides therapeutic effects similar to those of NSAIDs without causing the unwanted side effects. Therefore, selective COX-2 inhibitors have come into the spotlight for its detrimental roles in inflammation-related diseases.

As shown in this experiment, TH effectively reduced the expression of COX-2 and PGE2 content. The proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are small secreted proteins, which mediate and regulate immunity and inflammation.

TNF-α is a well-known pro-inflammatory cytokine with a wide range of biological functions. Bacterial LPS acts on macrophages to release TNF-α, and the secreted TNF or LPS then induces the cells to release IL-1β, IL-6 and IL-8. Interleukin-1 (IL-1) exhibits pro-inflammatory effects, especially by increasing synthesis of potent mediators and by up-regulating the expression of adhesion molecules on immune cells. IL-1β co-stimulates activation of T-cells, promotes maturation of B-cells, enhances NK activity, increases adhesion molecules expression. IL-6 is part of a family of cytokines that act through the gp130 receptor, and is an extremely important cytokine in the regulation of inflammation and immunity. IL-6 stimulates lymphocyte activation and proliferation.

Here, we evaluated the inhibitory effects of TH on LPS stimulated macrophages. Our results indicated that TH significantly inhibited LPS-induced TNF-α, IL-1β and IL-6 secretions.

Nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1) and CCAAT/enhancer binding protein (C/EBP) have been well defined, to be associated with iNOS and COX-2 expression. Among these, the NF-κB is a functionally transcriptional factor. NF-κB plays an important role in the regulation of immune and inflammation response, such as major histocompatibility complex I (MHC-I), MHC-II, interferon regulatory factor-1 (IRF-1), and diverse cytokines (TNF-α, IL-1, IL-6, IL-8 etc.).

In resting cells, NF-κB is sequestered in the cytoplasm as an inactive form through association with one of several inhibitory molecules like IκBa, IκBβ, IκBε, p105, p100. Activation of the signaling cascade of NF-κB (i.e., infection of virus or bacteria) results in a complete degradation of IκB or partial degradation of the carboxyl termini of p105 and p100 precursors. So the translocation of NF-κB to the nucleus induces transcription of COX-2, iNOS, Bcl-XL and cellular inhibitor of apoptosis proteins in nucleus. The kinds of IκB protein have been known to IκBa, IκBβ and IκBe. Among them IκBa is the most abundant inhibitory protein for NF-κB.

In our result, pretreatment of 0.1 and 0.3 mg/ml of TH reduced the LPS-induced p-IκBa expression level. These findings suggest that TH inhibited LPS-inducible IκB phosphorylation. Thus TH can prevent NF-κB translocation from cytosol to nucleus by inhibiting IκB phosphorylation.

In conclusion, we determined that the TH can have anti-inflammatory activity by suppressing the phosphorylation of IκB, and by inhibiting the expression of iNOS and COX-2 in LPS-activated Raw264.7 cells.

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References


